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Identification of putative motifs involved in the virulence of infectious pancreatic necrosis virus☆

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Abstract

Infectious pancreatic necrosis viruses (IPNVs) belonging to the family *Birnaviridae* display a high degree of antigenic variability, pathogenicity, and differences in outbreak mortality in salmonid species. To determine if virus isolates of Sp serotype differ in virulence, fry of Atlantic salmon (*Salmo salar* L.) were challenged with nine different field strains. These viruses caused either high mortality and severe pathological changes or low mortality and no lesions. To study the molecular basis for the variation in virulence of IPNV, complete nucleotide sequences of segment A of all these strains as well as segment B of three selected strains were determined. All viruses tested had a unique genome sequence. Only minor differences were noted in the genes encoding VP1, VP3, and VP4 proteins, whereas most changes were observed in the gene encoding the VP2 protein. A high level of variation was found in the small open reading frame (ORF), which encodes a 15-kDa nonstructural (NS) polypeptide also known as VP5. One of the strains lacked the initiation codon for this protein, whereas the other four could encode a truncated version of the NS protein. Additional data obtained by sequencing of the NS and VP2 genes directly from diseased fish demonstrated changes in the VP2 gene after two passages in cell culture, which could possibly be associated with attenuation. Comparison of the deduced amino acid sequences of the NS and VP2 genes reveals that the virulent strains possess a 12-kDa coding NS gene and have residues Thr, Ala, Thr/Ala, and Tyr/His at positions 217, 221, 247, and 500 of the VP2 gene, respectively—the motifs identified in this study to be involved in the virulence of IPNV.

Keywords: IPNV; Sequence; Genetic variation; Virulence; Sp serotype

Introduction

Infectious pancreatic necrosis virus (IPNV) is the causative agent of infectious pancreatic necrosis (IPN) in juvenile salmonid fish worldwide (Hill and Way, 1995). Most Norwegian isolates are of the Sp serotype (Melbye and Christie, 1994), also causing mortality in Atlantic salmon, *Salmo salar* L., post-smolts (Jarp et al., 1994; Smail et al., 1992). Clinical signs observed in diseased fish are distended abdomen, aberrant swimming, and darkened pigmentation, and focal necrotic lesions in the exocrine pancreatic tissue are detected by histopathological examination (McKnight and Roberts, 1976). Survivors are usually persistently infected (Mangunwiryo and Aguis, 1988; Reno et al., 1978). Mortality observed in an outbreak varies considerably and has been ascribed to differences in genetic susceptibility of the host (Ozaki et al., 2001; Silim et al., 1982) and differing level of environmental stress (Frantsi and Savan, 1971; McAllister and Owens, 1986). It is known both from experimental data (McAllister and Owens, 1986) and from practical experience with IPNV challenge studies that the virus tends to loose virulence after cell culture passage. The molecular basis for this attenuation has not been identified. The genomic variation between serotypes of IPNV is considered high compared to infectious bursal disease virus (IBDV) (Heppell et al., 1995b). There are indications of variation in virulence between isolates of the same serotype (Bruslind and Reno,

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2000; Shivappa et al., submitted for publication), but it is not conclusive if this is a general trait of IPNV.

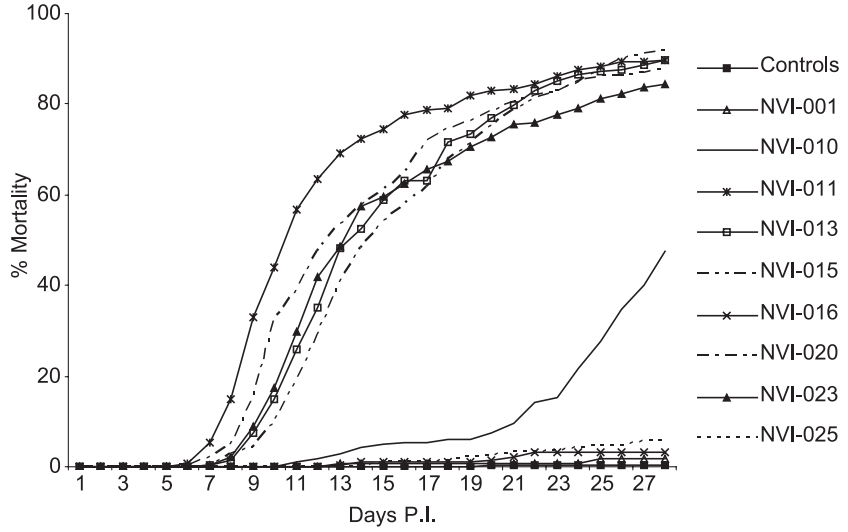
IPNV is the prototype virus of the *Birnaviridae*, a family of bisegmented double-stranded RNA viruses (Van Regenmortel et al., 2000). The smaller genome segment, segment B (2784 bp), is monocistronic and encodes a 94-kDa protein, VP1, which is the putative RNA-dependent RNA polymerase (Duncan et al., 1991). The larger genome segment, segment A (3097 bp), is bicistronic, the larger open reading frame (ORF) encodes a 107-kDa polyprotein (NH_2-preVP2-VP4-VP3-COOH) that is cotranslationally cleaved to generate the viral capsid proteins, VP2 and VP3, by the VP4-associated protease activity (Dobos, 1977; Duncan et al., 1987). Recent work has localized the protease cleavage sites of the polyprotein between amino acids 508 and 509 of the VP2–VP4 junction and between amino acids 734 and 735 of the VP4–VP3 junction (Petit et al., 2000). The small overlapping ORF is in a different reading frame and encodes an arginine-rich nonstructural (NS) polypeptide found in infected cells (Heppell et al., 1995a; Magyar and Dobos, 1994). However, there is no conclusive evidence that this protein is present in purified virions. It has recently been shown that the initiation of translation for the NS protein of VR-299 and Sp strains starts at the second in-frame start codon (Shivappa et al., submitted for publication; Weber et al., 2001), and the absence of expression does not influence virus growth in vitro (Weber et al., 2001). Evidently, there are natural strains that do not encode the NS protein (Heppell et al., 1995a, 1995b). The polypeptide, also called VP5, contains Bcl-2 homolog domains and overexpression of VP5 in CHSE-214 cells enhances cell viability during IPNV infection (Hong et al., 2002).

To determine the molecular basis of virulence in Norwegian IPNV serotype Sp strains, Atlantic salmon fry were challenged with nine different field isolates. The mortality and pathology caused by each isolate were recorded. The virulence of IPNV was previously shown to be associated with segment A (Sano et al., 1992), but has not been ascribed to specific motifs. To study the correlation between virulence and the virus genome, the complete nucleotide sequence of segment A from all viruses was obtained after two cell culture passages. The VP1 coding region of segment B was also determined for three selected viruses. The 5′ end of segment A, encoding VP2 and the NS protein, of four viruses was sequenced directly from diseased fish, the purpose being to reveal any cell culture adaptive changes in the viral genome. The investigation detected several motifs in VP2 and NS genes that correlated with virulence. Furthermore, the study shows that the viral genome changes rapidly after cell culture passage. It is important to be aware of these cell culture adaptive changes as they may have an affect on the outcome of challenge studies and on in vitro recovery of virus from cloned cDNAs.

Results

Fry challenge

The results of the fry challenge study are depicted in Fig. 1. Five of the nine isolates (NVI-011, NVI-013, NVI-015, NVI-020, and NVI-023) gave a mean cumulative mortality ranging from 84% to 92% and exhibited a similar course of mortality. The fish mortality started 7 days post challenge and peaked at 10–12 days. The fish exhibited classical clinical signs of IPN; darkened pigmentation, aberrant swimming, and ascites. For one of the isolates (NVI-010), the mortality was low until the last week of the challenge experiment, but then it rapidly increased to give a mean cumulative mortality of 48% (range: 21–64%).



Isolate	Percent cumulative mortality (Mean (SD))
Controls	0,5 (1,0)
NVI-001	1,5 (1,9)
NVI-010	48 (19,4)
NVI-011	90 (6,9)
NVI-013	90 (3,7)
NVI-015	92 (9,6)
NVI-016	3 (1,2)
NVI-020	88 (3,4)
NVI-023	84 (7,9)
NVI-025	6 (7,2)

Fig. 1. Percent cumulative mortality of Atlantic salmon fry challenged with nine IPNV serotype Sp isolates. The average mortality in four tanks of approximately 47 fish is calculated for each virus isolate, and the variation among tanks is expressed as the standard deviation, as shown in the table.

Three isolates, NVI-001, NVI-016, and NVI-025, caused only minor mortalities (Fig. 1).

Only one of the control fish died during the challenge period. IPNV could not be isolated from control fish or from fish sampled before challenge (seven fry examined). The virus was readily reisolated (minimum three fish out of four positive) from fish challenged with all isolates except NVI-001. For isolate NVI-001, 20 additional fish were analyzed but none were found positive for IPNV.

Histopathology and immunohistochemistry

The highly virulent isolates causing approximately 90% mortality (NVI-011, NVI-013, NVI-015, NVI-020, and NVI-023) displayed similar histopathological changes, and hence the results are presented together. Focal necrotic lesions were first observed in the livers of fish sampled 5 days postinfection (PI) and in the exocrine pancreas and gastric glands of fish sampled 7 days PI. From 9 days onwards, the lesions in both liver and exocrine pancreas were graded as severe with a massive coagulation necrosis. No lesions were detected in fry challenged with isolates causing less than 10% mortality (NVI-001, NVI-016, NVI-025) or in the group infected with NVI-010. No histomorphological changes were found in control fish or in fish sampled before challenge.

In the high-mortality groups, the viral antigen was first detected by immunohistochemistry in the liver and pancreas 3 days after challenge and in the gastric glands and kidney at day 7 and onwards. Positive immunostaining for IPNV was demonstrated both intracellular and in extracellular cell debris in the pancreas, liver, and gastric glands (Fig. 2). In the kidney, it was mainly localized inside large leucocytes resembling macrophages (not shown). Fish sampled during the peak of mortality had an extensive viremia, as observed by strong immunostaining in the lumen of vessels (Fig. 2D). No viral antigen was detected in control fish, fish sampled before challenge, fish challenged with NVI-010, or strains causing less than 10% mortality.

Sequence analysis of IPNV genomes

The complete nucleotide sequence of IPNV segment A of all nine isolates was determined, including the 5′ and 3′ terminal regions, by direct sequencing of the DNA of reverse transcription (RT)-PCR products. Comparison of the genomic relationship of the VP2 protein sequence for

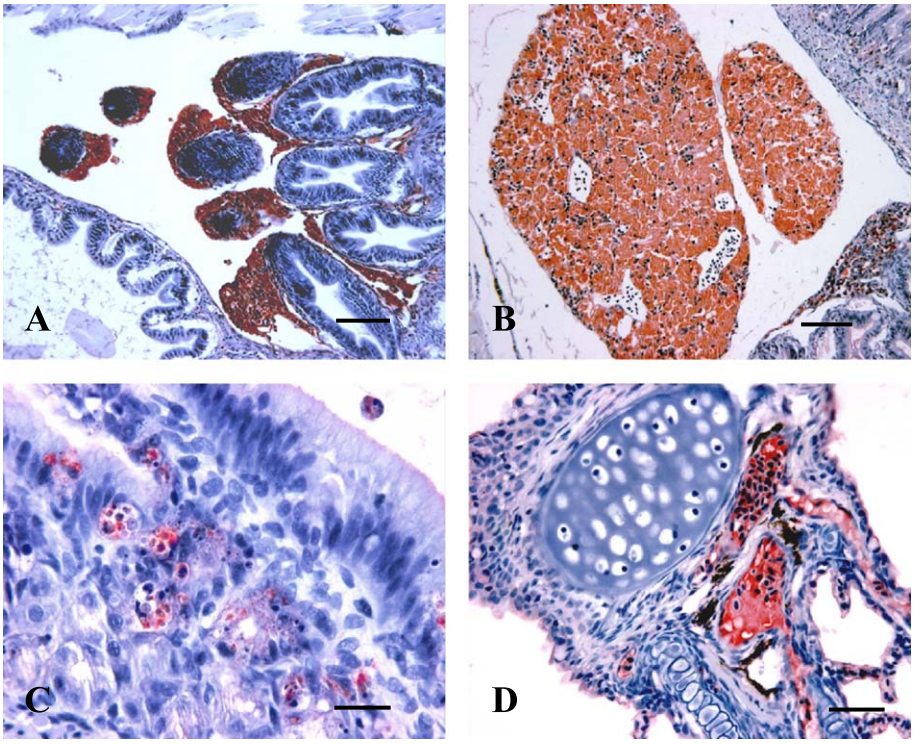


Fig. 2. Immunohistochemical detection (red color) of IPNV in challenged Atlantic salmon fry using a polyclonal antibody against Sp serotype. (A) Pancreas from fry challenged with isolate NVI-013, 9 days postinfection (PI). A diffuse staining of the exocrine pancreatic tissue is observed. Scale bar = 100 μm. (B) Liver from fry challenged with isolate NVI-020, 9 days PI. There is strong diffuse immunostaining and massive destruction of the liver parenchyma. Scale bar = 100 μm. (C) Gastric mucosa of fry challenged with isolate NVI-011, 7 days PI. Positive immunostaining is localized to necrotic lesions in the gastric glands. Scale bar = 25 μm. (D) Gill section from fry challenged with NVI-020, 11 days PI. Viral antigen is detected in the blood vessel lumina at the base of the primary lamella. Scale bar = 40 μm.

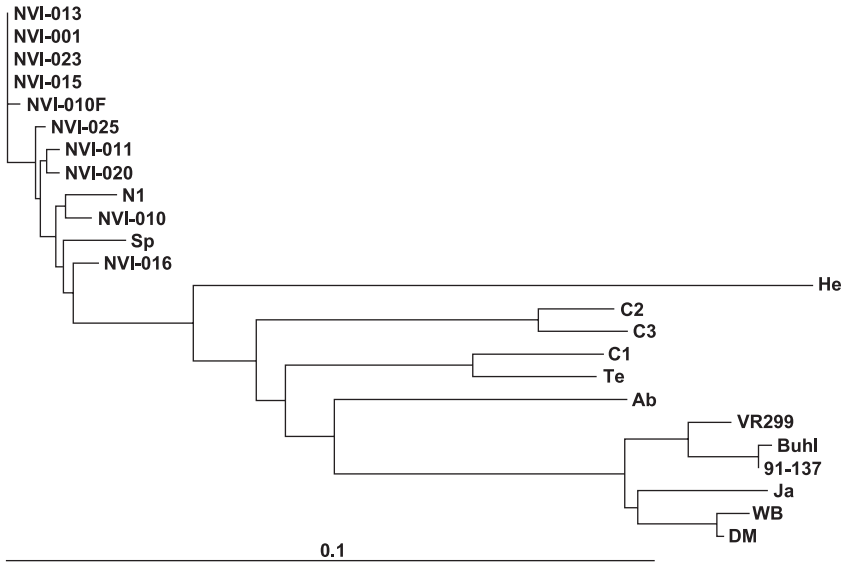


Fig. 3. Phylogram of VP2 protein sequences of isolates included in this study aligned with previously published IPNV VP2 sequences (Blake et al., 2001). The isolates cluster with the Sp serotype and are related to the Norwegian N1 isolate.

the viral isolates included in this study to previously sequenced IPNV isolates (Blake et al., 2001) shows the closest relationship to the Norwegian N1 strain of the Sp serotype (Fig. 3). Comparison of the nucleotide and deduced amino acid sequence between the isolates revealed little variation in the noncoding region or in the VP3 (98.3% amino acid identity) and VP4 (99.1% amino acid identity) coding regions. A large part of this variation was caused by isolate NVI-016 alone (two unique changes in both VP3 and VP4), which is more distantly related to the rest of the isolates (Fig. 3). Sequence comparison of the VP1 coding region of segment B from isolates NVI-013, NVI-015, and NVI-016 demonstrated a high degree of homology (99.8% amino acid identity).

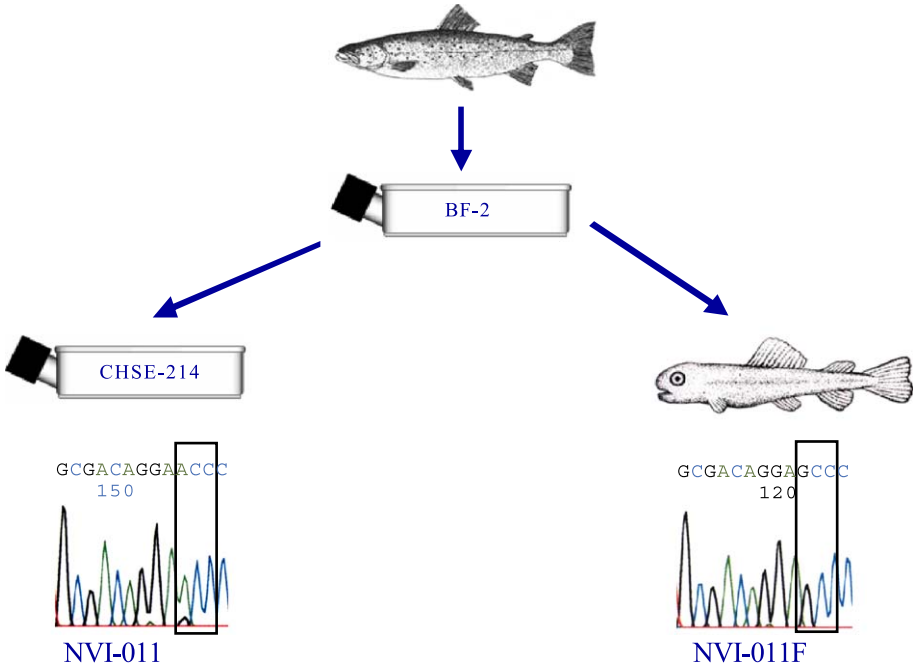


Fig. 4. The sequence of isolate NVI-011 derived from tissue culture and from fish. The sequence of isolate NVI-011 was first obtained after two cell culture passages. At nucleotide position 779, encoding amino acid 221 of VP2, A/G double peaks were observed (left chromatogram). Sequencing of the NS and VP2 encoding region directly from diseased fish (NVI-011F) revealed only one nucleotide difference, a single G peak at nucleotide position 779 (right chromatogram).

The variation in the VP2 coding region was mainly confined to amino acid residues 217, 221, 247, and 500 (Table 1A). At position 221, the chromatograms often displayed double peaks (A/G at nucleotide 779). To check if this was a cell culture-induced phenomenon, the nucleotide sequence of NS and VP2 genes of isolates NVI-011, NVI-015, and NVI-020 were determined directly from diseased fish (Fig. 4). The only difference was that all fish-derived viral genome sequences had only alanine at position 221 in VP2 as compared to a mixture of threonine–alanine from tissue culture-derived material (Table 1B).

From analysis of possible open reading frames, the translation of the NS protein for all the isolates should start at nucleotide 112. The first stop codon in this ORF normally appears at nucleotide 511, resulting in a polypeptide of 133 amino acids. Only four isolates analyzed followed this general pattern (NVI-001, NVI-016, NVI-020, and NVI-025) (Table 1A). NVI-011 had a premature stop codon at nucleotide 496, encoding a polypeptide shorter by five amino acids. NVI-013, NVI-015, and NVI-023 could encode a truncated NS protein of 105 amino acids, having a stop codon at nucleotide 427. Interestingly, all isolates encoding truncated NS proteins were highly pathogenic. Isolate NVI-010 has a mutation in the start codon at nucleotide 112 that would ablate the expression of this protein.

Groups of fish challenged with isolate NVI-010 had a delayed onset of mortality. The sequence of this isolate was also obtained directly from viral RNA isolated from diseased fish. The virus recovered at the end of the challenge study (NVI-010F) had a different amino acid composition and had a full complement of the NS protein (133 amino acids) (Table 1B).

Table 1A
Variation in the NS gene and in the VP2 coding region of nine IPNV field isolates, sequences obtained after two cell culture passages[a]

Isolate[b]	NS				VP2			
	ATG 112	TGA 427	TGA 496	TGA 511	217	221[c]	247	500
NVI-001	x			x	Thr	Ala/Thr	Thr	Tyr
NVI-010				x	Pro	Thr	Ala	His
NVI-011	x		x		Thr	Thr/Ala	Ala	His
NVI-013	x	x			Thr	Ala/Thr	Thr	Tyr
NVI-015	x	x			Thr	Ala/Thr	Thr	Tyr
NVI-016	x			x	Pro	Thr	Ala	Tyr
NVI-020	x			x	Thr	Thr/Ala	Ala	His
NVI-023	x	x			Thr	Ala/Thr	Thr	Tyr
NVI-025	x			x	Pro	Ala	Ala	His

[a] This table shows variation possibly correlated with virulence; some additional minor variations in the NS or VP2 sequence are not included.
[b] The isolates causing high mortality are written in bold types.
[c] Chromatograms from sequences obtained after two cell culture passages often had a double peak at VP2 residue 221, representing a mix of two amino acids. The dominating amino acid is named first in this table.

Table 1B
Variation in the NS gene and in the VP2 coding region of four IPNV field isolates, sequences obtained directly from challenged and diseased fish[a]

Isolate	NS				VP2			
	ATG 112	TGA 427	TGA 496	TGA 511	217	221	247	500
NVI-010F	x			x	Thr	Ala	Thr	Tyr
NVI-011F	x		x		Thr	Ala	Ala	His
NVI-015F	x	x			Thr	Ala	Thr	Tyr
NVI-020F	x			x	Thr	Ala	Ala	His

[a] This table shows variation possibly correlated with virulence; some additional minor variations in the NS or VP2 sequence are not included.

Discussion

Despite of the worldwide distribution of IPNV and its high economical impact on modern aquaculture, little information is available on virulence variation between serotypes and within serotypes. In this study, we have shown that IPNV strains of the same serotype differ in virulence and display heterogeneity in the part of the viral genome encoding the structural protein, VP2, and the nonstructural polypeptide (VP5).

Sequence analysis of nine IPNV strains revealed more variation in the VP2 coding region compared to VP1, VP3, or VP4. VP2 is the major outer viral capsid protein and the majority of epitopes recognized by neutralizing antibodies are located on VP2 (Frost et al., 1995; Tarrab et al., 1995). Heppell et al. (1995b) identified two short hypervariable segments within the central region of the VP2 coding region based on a study of seven virus strains. This variable domain corresponding to amino acid residues 243–335 was confirmed in a study of 28 isolates by Blake et al. (2001). However, only one (247) out of four highly variable VP2 amino acids (217, 221, 247, 500) detected in this study is located within the hypervariable region. At position 217, threonine was associated with virulence in this study, while proline at this position was found only among the low virulent isolates. Only NVI-001 did not follow this general pattern, but because the virus could not be reisolated from any of the 24 fish examined, we do not know if the challenge with this virus was actually successful. In the study by Blake et al. (2001), only 1 out of 24 strains had threonine at position 217, while proline is found in 14 strains. Bruslind and Reno (2000) also found threonine in the most virulent Buhl isolate, while the two other less virulent strains had alanine at this position. Taken together, these findings point towards residue 217 as being of key importance for explaining variation in IPNV virulence.

Most of the isolates sequenced in this study were passaged two times in cell culture, and their chromatograms showed two peaks at nucleotide position 779, which would code for both alanine and threonine residues at position 221.

The presence of only alanine in all four isolates sequenced directly from diseased fish shows that the amino acid at position 221 changes rapidly from alanine to threonine after a few cell culture passages. This cell culture adaptation may be involved in attenuation, and studies on the impact of this specific amino acid substitution on virulence are in progress. It is interesting to note that almost all previously published sequences of IPNV strains have threonine at position 221 and that very few of these sequences, as far as we know, originate from viral RNA obtained directly from diseased fish. The amino acid residues at position 247 and 500 vary, but do not seem to be directly correlated with virulence because virulent isolates have both alanine–threonine and histidine–tyrosine at positions 247 and 500, respectively.

An unexpected finding was the variation in the ORF coding for the nonstructural polypeptide (NS). From previously sequenced IPNV strains, only the Hecht isolate is known to lack an open reading frame for the NS protein (Heppell et al., 1995a). In this study, isolate NVI-010 did not encode the NS protein. The mortality curve of this strain was atypical as the onset of mortality was 2 weeks later than for the virulent strains. Further to this, the nucleotide sequence of the virus recovered from diseased fish (NVI-010F) was different from the original strain (NVI-010). This could possibly result from contamination from one of the other tanks, but the same phenomenon was observed in four randomly placed parallel tanks, and the recovered isolate was different from the other virulent strains as it had a unique nucleotide sequence not found in any other strains included in this trial. In the NVI-010 tank reserved for tissue sampling, no mortality was recorded, and no pathological changes were observed in fish sampled from this tank. However, the sampling may have interfered with the spread of virus between individuals in the tank and thus mortality. Also at the onset of mortality (by day 21) few fish were left in this tank. The possibility of the initial NVI-010 strain used for infection was a combination of a NS-negative strain together with a virulent isolate (NVI-010F) encoding a full-length NS protein cannot be excluded, and the only way to decide this is to plaque purify isolate NVI-010. The virulence of the NS mutant is not known, but it is interesting to note that four of the five virulent isolates encoded truncated NS proteins. Recently, Hong et al. (2002) documented an anti-apoptotic activity from overexpressing the NS protein (also called VP5) in cell culture. Expression of a BH2 domain deletion variant caused loss of the cell viability-enhancing ability (Hong et al., 2002). The truncated NS variants encoded by isolates NVI-013, NVI-015, and NVI-023 would correspond to a BH2 domain deletion variant, but it is not known if loss of the anti-apoptotic activity would influence virus virulence in vivo. Moreover, some of the highly virulent isolates encode the full-length NS, and thus it is not possible to draw firm conclusions on the importance of the NS protein in virulence. Nevertheless, the finding of four different NS variants among eight IPNV isolates collected within the same geographical region underlines the need of more information on the function of this protein and its role in viral virulence. Weber et al. (2001) used reverse genetics technique to reveal that the absence of this NS protein does not influence viral replication in cell culture. The same approach should be pursued for studying the function of the protein on viral virulence in vivo.

Characteristic for RNA viruses in general is the absence or the low efficiency of proofreading activities associated with RNA polymerases, leading to a high mutation rate during genome replication. As a consequence, RNA viruses are thought to replicate as complex and dynamic mutant swarms called viral quasispecies (Domingo and Holland, 1997). There are evidence pointing toward both IPNV (Hsu et al., 1995) and IBDV (Jackwood and Sommer, 2002) existing as quasispecies. The apparent heterogeneity at VP2 residue 221 after two cell culture passages may be a result of a selection of cell culture-adapted clones that are present as a minor fraction of the virus population in the diseased fish.

The fry challenge also shows that IPNV can use both the acute and the persistent patterns of host infection (Villarreal et al., 2000). Highly virulent strains like NVI-011, NVI-013, NVI-015, NVI-020, and NVI-23 exhibited mortality of approximately 90% of susceptible fry and induced severe pathological lesions, whereas fish infected with closely related isolates like NVI-016 and NVI-025 gave low percentage of mortality. The absence of lesions combined with the fact that the virus was readily reisolated from infected fish 28 days after challenge suggests that the low-virulent strains survive in the fish population by using a persistence strategy. The anticipation is that IPNV would trade-off an efficient replication and transmission for extended survival in the host. Such a strategy would be more suitable in a situation where the number of animals available for infection would be very low, like in a natural ocean situation with low population densities. In contrast, the high population density in aquaculture favors an acute virus life strategy or efficient transmission and selection, thus would be in direction of more virulent strains exploiting unrestricted host-to-host spread. More information is needed to understand how these closely related strains with differing virulence characteristics interact in a disease situation.

Lesions develop very rapidly in fish challenged with virulent isolates. The pancreas and liver lesions were generally graded as mild on days 5 and 7 post infection, while 9 days after challenge, the majority of fish sampled had severe lesions and widespread viral antigen in both liver and pancreas. The massive liver damage is commonly seen in diseased fry from IPN field outbreaks and has also been reported from IPNV fry challenge studies in Norway (Taksdal et al., 1997). It is not known whether IPNV strains other than the Norwegian Sp strains can cause substantial liver pathology. To our knowledge, this is the first report of IPNV-induced lesions in the gastric glands of salmonid fish. Necrosis of gastric glands has been reported in Japanese eels infected with an aquatic birnavirus, serologically related to

IPNV Sp (Lee et al., 1999), but not in other fish species. The pancreas, liver, and gastric glands have ontogenetically a common origin as they all develop from the embryonic foregut. The possibility of these cells carrying common features, like virus attachment molecules or receptors, might point to these cells being more susceptible to infection than other cells. Both gills and intestines have been suggested as possible routes of entry for the virus. IPNV is very resistant to acid pH and is shown to survive passage through a cow's gut (Smail et al., 1993a, 1993b). This gives reason to believe that the virus can pass the stomach of the fry undamaged, and the gastric glands may represent a possible route of infection for the virus. In this study, the lesions in the gastric glands and the detection of virus by immunohistochemistry generally appeared at a later time than for liver and pancreas. Nevertheless, an initial low level of viral replication in the gastric glands cannot be ruled out.

By sequencing nine IPNV isolates of known virulence, we have revealed several motifs possibly correlated with virulence. Isolates with truncated NS proteins are generally virulent, and threonine at amino acid residue 217 and alanine at position 221 of VP2 are characteristic for the virulent IPNV serotype Sp strains studied. The implication of the NS protein length and the VP2 amino acids should be studied independently, preferably using the reverse genetics approach.

Materials and methods

Fry challenge

Bluegill fry cells (BF-2, ATTC No. CC 91) were used for the detection, propagation, and quantification of the virus. The cells were grown at 20 °C in Eagle's minimum essential medium with Earle's balanced salt solution (EBSS) supplemented with 10% fetal bovine serum (FBS) and 50 μg ml^{-1} gentamicin. For virus propagation, the incubation temperature was 15 °C.

The viruses used in this study were isolated from fish tissue samples collected from field outbreaks of IPN in northwestern Norway from May to August 2000. Veterinarians had diagnosed IPN based on clinical observation, autopsy, and by use of an agglutination test (Taksdal and Thorud, 1999). The National Veterinary Institute had confirmed the diagnosis after histopathological and immunohistochemical examination. The material originated from both fresh- and seawater-reared Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*), and the mortality recorded in the field outbreaks varied from 2% to 70%. The samples were stored at −20 °C until preparation of a tissue homogenate (10%, w/v, in EBSS added 50 μg ml^{-1} gentamicin). They were screened for the presence of IPNV by inoculation of BF-2 cells, and the results were confirmed by a neutralization test with a polyclonal antibody towards IPNV serotype Sp. All specimens were positive for IPNV. Before challenge, eight isolates were propagated by one passage in BF-2 cells. The cell culture supernatant was obtained after a brief centrifugation, and the infectious titer was determined by end point dilution of on BF-2 cells grown in 96-well plates. The $TCID_{50}$ was estimated by the method of Kärber (1931). One additional isolate (Sp103, GenBank accession numbers , AY354519 and , AY354520), previously causing low mortality in challenged fish (Shivappa et al., submitted for publication), was also included in the study. It was renamed NVI-025 as nucleotide sequencing results detected one nucleotide change at position 2765 resulting in an amino acid substitution from tyrosine to aspartate at residue 149 of VP3.

Atlantic salmon fry with an average weight of 0.16 g at onset of feeding were kindly provided by Aqua Gen AS, Sunndalsøra, Norway. The fry were full siblings, originating from parents that came from a family line known to be susceptible to IPNV infection. There had been no records of disease in the hatchery. As the fry arrived at the research station, they were allowed to acclimatize for 1 week before challenge. Throughout the challenge, fish were held in freshwater tanks with a total water volume of 8 l. The temperature was 10 °C and the oxygen concentration minimum was 8 mg l^{-1} at outlet.

A total of 2585 fry were included in the challenge. At arrival, they were distributed into 10 tanks, each of approximately 235 fry. The fish were deprived of food the day before challenge, which was carried out by bath exposure at a dose of 10^4 $TCID_{50}$ ml^{-1}, for each of the nine IPNV isolates. One tank of control fish was exposed to cell culture medium only. The day after challenge, fry from each of the 10 tanks were divided into five subgroups of 47 each and held in randomized separate tanks for the next 30 days.

On days 1, 3, 5, 7, 9, 11, 13, 15, 18, 21, 25, and 29 following challenge, three fry from tank 1 (challenge tank) were humanely killed and submerged in excess volumes of buffered formalin. Only live fish, preferably with clinical signs, such as aberrant swimming or ascites, were sampled for formalin fixation. The mortality in the tanks reserved for sampling was not included when calculating the mean cumulative mortality. Tanks 2, 3, 4, and 5 were placed randomly and used for recording mortality on a daily basis. Dead fish were collected each day and frozen at −70 °C. Some of these fish were later used for virological examination by inoculating the tissue homogenate (10%, w/v, in EBSS added 50 μg ml^{-1} gentamicin) onto BF-2 cells. The samples were considered as negative when no cytopathic effect was observed after two passages. The same procedure was employed to check if the virus could be recovered from control fish and from fish sampled before challenge.

Histopathology and immunohistochemistry

All samples of formalin-fixed fry were processed and embedded in paraffin wax according to standard procedures.

Sagittal sections of whole fry were routinely stained with hematoxylin and eosin (HE) and examined by light microscopy. Duplicate sections were examined by immunohistochemistry to detect IPNV. The sections were processed according to a method described by Evensen and Olesen (1997). After blocking using bovine serum albumin (BSA), the slides were incubated with a rabbit antiserum specific for IPNV (Evensen and Rimstad, 1990) at a dilution of 1:1000 for 30 min. The incubation with the secondary antibody (biotinylated anti-rabbit immunoglobulin diluted 1:300) was for 30 min, followed by streptavidin-biotin alkaline phosphatase diluted 1:1000 for 1 h at 37 °C. After washing, Fast red (1 g l^{-1}; Sigma, St Louis, MO, USA) and Naphthol AS-MX-phosphate (0.2 g l^{-1}; Sigma) with 1 mM levamisole (Sigma) in 0.1 M TBS were added to develop for 20 min. Incubations were carried out at room temperature unless stated otherwise. Sections were counterstained with Mayer's hematoxylin and mounted in aqueous mounting medium (Aquamount; BDH Laboratory). The histopathological changes of the pancreas, liver, and gastric glands were scored as absent, mild, moderate, or severe. The immunohistochemical labellings of the pancreas, liver, kidney, and gastric glands of each fry were scored as absent, infrequent, common, or widespread.

Cloning and sequencing of the IPNV genome

Viral genome sequencing was performed as described previously with slight modifications (Yao and Vakharia, 1998). CHSE-214 cells (ATCC CRL-1681) were maintained at 20 °C in minimal essential medium containing Hank's salts and supplemented with 10% fetal bovine serum (FBS). CHSE cells were infected with the virus supernatant used in the fry challenge and kept at 15 °C. CHSE cells were used rather than BF-2 cells to avoid selection of certain clones adapted to this specific cell line. When widespread cytopathic effect was visible, cell debris was pelleted at 5000 × *g* for 30 min at 4 °C. The supernatant was centrifuged at 100 000 × *g* for 2 h at 4 °C to pellet the virus before digestion with proteinase K (100 μg ml^{-1} final concentration) for 3 h at 37 °C. Viral RNA was isolated by phenol-chloroform extraction and ethanol precipitation and used for reverse transcription (RT)-PCR using a kit in accordance to the suppliers protocol (Perkin-Elmer). Segment A was amplified using two primer pairs (A-A5''NC plus A-SpKpnR, A-SpKpnF plus A-SpPstR) to produce two overlapping cDNA fragments (Table 2). Segment B was also amplified as two overlapping fragments using primers B-B5'NC and B-SpBIR for the 5′ half and primers B-SpBIF and B-Bgl3'NC for the 3′ half. To determine the 3′ and 5′ end of segment A, the viral RNA was polyadenylated and reverse transcribed with a poly(dT) primer and resulting cDNAs were amplified by PCR using primer A-BstER for the 5′RACE and A-Sp3′F for the 3′RACE. The RT-PCR products were separated by agarose gel electrophoresis, purified using QIAquick gel extraction kit (Qiagen), and directly sequenced by the dideoxy chain termination method in an automated DNA sequencer (Applied Biosystems) using sequence-specific primers. The sequence data were analyzed using PC/Gene (Intelligenetics) software. Phylogenetic analyses of the sequenced isolates and previously sequenced isolates were carried out using the ClustalX program (Thompson et al., 1997) and the phylogram was printed using the TreeView software (Page, 1996).

To obtain the viral genome sequence directly from diseased fish, the same procedures were used, except that the original material was tissue homogenate (1:10, w/v) in cell culture medium without FBS. Primer pairs A-A5'NC and A-Sp1689R were used to generate a 1689-bp PCR product covering the NS and VP2 coding region of segment A, the PCR product was used for direct sequencing.

Table 2
Oligonucleotides used for RT-PCR and sequencing of IPNV serotype Sp segments A and B[a]

Nucleotide sequence	Orientation	Designation	Nucleotide no.
*TAATACGACTCACTATA*GGAAAGAGAGTTTCAACG	+	A-A5'NC	1–18
GGCCATGGAGTGGTACCTTC	–	A-SpKpnR	1584–1603
GAAGGTACCACTCCATGGCC	+	A-SpKpnF	1584–1603
AAAGCTTCTGCAGGGGGCCCCCTGGGGGGC	–	A-SpPstR	3079–3097
GAGTCACAGTCCTGAATC	+	A-Sp500F	597–614
CAAGGATGGTATTCACCG	+	A-Sp1970F	2067–2084
AGCCTGTTCTTGAGGGCTC	–	A-Sp1689R	1671–1689
GAACAAAATCAGACGTCTAGCC	+	A-Sp3'F	2764–2785
*TAATACGACTCACTATA*GGAAACAGTGGGTCAACG	+	B-B5'NC	1–18
GTTGATCCCCGTCTTTGCTTCG	–	B-SpBIR	1643–1622
CTTCCTCAACAACCATCTCATG	+	B-SpBIF	1529–1550
AAGATCTGGGGTCCCTGGCGGAAC	–	B-Bgl3'NC	2766–2783
CAACAGGCTACTGGCAATGAAG	+	B-Sp620F	605–626
GTAAGGTATGAAGCGATTCGGCT	+	B-Sp1900F	1932–1954

[a] Virus-specific sequences are underlined. The primers used to make a clone of the 5' end of segment A and B contain a T7 promotor sequence written in italic type. The positions where the primers bind (nucleotide number) are in accordance with the published sequence of the West Buxton strain (Yao and Vakharia, 1998).

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